Incursion and Spread of H5N1 Highly Pathogenic Avian Influenza Viruses among Wild Birds in 2010–11 Winter in Japan

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ABSTRACT. Many highly pathogenic avian influenza (HPAI) outbreaks occurred in Japan during the 2010–11 winter. H5N1 HPAI viruses were isolated from 63 wild birds including migrating and resident birds, and caused HPAI outbreaks in 24 chicken farms by the end of March. In the present study, all virus strains isolated from wild birds in western Japan together with the viruses in the preceding works were phylogenetically and epidemiologically analyzed. Furthermore, the virus distributions in the raptors that died of H5N1 HPAI virus infection were assessed. The virus isolates in Japan were classified into three groups by phylogenetic analysis of their hemagglutinins, supporting the previous report (Sakoda *et al.*, 2012). The viruses in each group were continuously isolated in respective limited areas, indicating that viruses were maintained in local bird populations throughout the outbreak periods. Some viruses were genetically closely related to the Korean isolates around the same periods, suggesting that migratory birds were suspected of contributing to transportation of the viruses across the sea. Viruses were recovered from systemic tissues including digestive organs of the deceased raptors, indicating that they were infected with HPAI viruses by their predatory behavior, eating infected birds or carrion in the environment.

KEY WORDS: H5N1, influenza virus, Japan, wild bird.

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Outbreaks of highly pathogenic avian influenza (HPAI) caused by H5N1 viruses have occurred in many countries, leading to serious economic losses in the poultry industry. In addition, the H5N1 HPAI virus (HPAIV) returned to the natural host, migratory water birds, and spread to Asia, the Middle East, Europe and Africa [2, 11]. These incidents have increased the possibility of further spread of HPAIVs to humans and rare species of animals.

In Japan, the outbreaks caused by H5N1 HPAIVs occurred in chicken farms in 2003–2004 [12]. H5N1 HPAIVs were also isolated from wild birds, such as jungle crow (*Corvus macrorhynchos*), mountain hawk-eagle (*Spizaetus nipalensis*) and whooper swan (*Cygnus cygnus*) in 2004, 2007 and 2008, respectively [17, 19, 20]. Furthermore, many outbreaks occurred in Japan during the 2010–11 winter. H5N1 HPAIVs were isolated from 63 wild birds including migrating and resident birds, and caused HPAI outbreaks in 24 chicken farms by the end of March [15, 21]. These outbreaks were controlled by depopulation of chickens in the relevant farms,

intensive surveillance and improved biosecurity measures.

In Japan, definitive diagnoses of HPAI in poultry are given by the National Institute of Animal Health (NIAH: Tsukuba, Japan). The wild birds with suspected HPAI receive diagnostic confirmation by Hokkaido University, NIAH or Tottori University. Which institute is selected for diagnosis depends on the area where HPAI occurs. After the HPAI outbreaks in 2010–11, the three institutes published a report [15]. Thirty-three isolates were obtained and were divided into three (A–C) groups based on the phylogenetic analysis of the hemagglutinin (HA) genes [15]. The report was based on the analyses of representative virus strains. Therefore, follow-up studies were essential to elucidate the reason why the massive HPAI occurred, and to prevent outbreaks in future. In the present study, all virus strains isolated at Tottori University together with the viruses in the preceding works [7, 15, 21] were phylogenetically and epidemiologically analyzed. Furthermore, the virus distributions in the deceased raptors were examined to evaluate their potential to be sources of infection for other bird species.

MATERIALS AND METHODS

Isolation and identification of viruses: The viruses were isolated from tracheal and cloacal swabs of wild birds. Swabs were collected in 2 ml of transport medium containing minimum essential medium (Nissui Pharmaceutical, To-

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kyo, Japan) with 10 mg streptomycin (Meiji Seika Pharma, Tokyo, Japan) and 10,000 units of penicillin G (Meiji Seika Pharma) and inoculated into the allantoic cavities of ten-day-old embryonated chicken eggs. After incubation at 35 °C for 48 hr, the allantoic fluids were harvested, and their HA activities were examined [16]. Subtypes of the HA and neuraminidase (NA) of influenza virus isolates were identified by polymerase chain reaction (PCR) and sequencing. Viral RNA was extracted from chicken embryos infected with viruses using QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) and reverse transcribed with the Uni12 primer [6] and PrimeScript reverse transcriptase (Takara, Otsu, Japan). The full length of HA and NA gene segment was amplified by PCR with gene-specific primers as described previously [6]. Each gene segment was directly sequenced with 3,130 Genetic analyzer (Applied Biosystems, Foster City, CA, U.S.A.). The subtypes of the viruses were determined by comparing their sequences with those of reference strains.

Phylogenetic analysis: To assess the genetic relationship among H5N1 HPAIVs isolated in the 2010–11 winter in Japan, the full nucleotide sequences of HA coding region were applied for phylogenetic analysis. The sequences were aligned by BioEdit software [5]. Phylogenetic trees were constructed by the neighbor-joining method [14] using MEGA 5 software [18]. Bootstrap values were calculated from 1,000 replicates.

Virus detection in the carcasses: Five raptors that died of H5N1 HPAIV infection were provided by each municipal animal health laboratory under the permission of Agency for Cultural Affairs, Ministry of Education, Culture, Sports, Science and Technology in Japan. Autopsies were conducted in a biosafety level 3 facility at Avian Zoonosis Research Center, Tottori University, Japan. Each tissue was aseptically collected and homogenized to make 10% (w/v) suspensions in nutrient broth medium with 10 mg streptomycin and 10,000 units of penicillin G and inoculated into the allantoic cavities of ten-day-old embryonated chicken eggs. The infectious viral titers in the collected samples were determined in ten-day-old embryonated chicken eggs by the method of Reed and Muench [13].

RESULTS

Virus isolation from wild birds: Sixty-three H5N1 HPAIVs were isolated from wild birds, and 24 H5N1 HPAIVs were isolated from chickens during 2010–11 outbreaks in Japan [15, 21]. Of these, 42 viruses were isolated at Tottori University from 12 species of dead wild birds from December 2010 to March 2011 (Table 1). The 12 species were Tundra swan (*Cygnus columbianus*), Hooded crane (*Grus monacha*), Common pochard (*Aythya ferina*), Tufted duck (*Aythya fuligula*), Black headed gull (*Chroicocephalus ridibundus*), Little grebe (*Tachybaptus ruficollis*), Mandarin duck (*Aix galericulata*), Peregrine falcon (*Falco peregrinus*), Ural owl (*Strix uralensis*), Mute swan (*Cygnus olor*), Grey heron (*Ardea cinerea*) and Great crested grebe (*Podiceps cristatus*).

Phylogenetic analysis of the HAs of the isolates: A phylogenetic tree of the HAs of all the isolates from wild birds

and chickens in Japan and Korea in 2010–11 winter [8, 21] is shown in Fig. 1. Group A consisted of 15 isolates from *Anseriformes* in Fukushima and Hokkaido prefectures (Fig. 1a). The viruses isolated from resident birds and chickens were not included in this group. The viruses isolated at Tottori University shown in Table 1 were classified into Groups B and C (Fig. 1a and 1b). Group B contained three isolates from raptors in addition to 14 isolates from waterfowl. Group C included the other 33 isolates in Japan (Fig. 1b). The HAs of the viruses isolated in Korea also belonged to this group. In Group C, several characteristic subgroups were observed: Subgroup C-1 consisted of the viruses isolated in Miyazaki and Oita prefectures, located in east area of Kyusyu region of Japan (see Fig. 2); Subgroups C-5 and C-6 were composed of the strains isolated in Kagoshima and Miyazaki prefectures, respectively; Subgroups C-2, C-3 and C-4 contained the viruses which were isolated in the south-middle area of Korea [9] in addition to those in Yamaguchi prefecture and the Kyusyu region of Japan.

Epidemiology: The locations and times of HPAI outbreaks in wild birds and chickens are shown in Fig. 2. Throughout the outbreaks, the viruses in Group A were isolated in limited areas of Hokkaido and Fukushima prefecture in northern Japan. The viruses in Group B were isolated in the mid-west area of Japan; the viruses were mainly perpetuated during outbreaks in the San-in area consisting of Tottori and Shimane prefectures, and the south part of Hyogo prefecture. The viruses in Group C were first found in fecal samples of ducks at Ohnuma lake, Hokkaido [7]. Then, the viruses were isolated from wild birds and chickens predominantly in the Kyusyu region until March, 2011. The outbreaks were frequent in Miyazaki and Oita prefectures, located at the east side of the Kyusyu region. In the late phase of the outbreaks, the viruses in Group C were isolated in the south and mid-east parts of the Honsyu region (e.g., Wakayama and Tochigi prefectures, respectively).

Virus recovery from birds that died of H5N1 HPAIV infection: Many viruses were isolated from raptors, such as peregrine falcon and northern goshawk (Table 1 and Fig. 1), while the populations of these bird species were smaller than those of water birds. Viruses were recovered from the systemic tissues of raptors (Table 2). In particular, viruses were detected in brain, lung, colon and cloaca of all raptors, and the viral titers in brain were highest among the tested tissues.

DISCUSSION

In the present study, all H5N1 HPAIVs isolated in Japan in the 2010–11 outbreaks were phylogenetically and epidemiologically analyzed. The viruses in Groups B and C had been mainly isolated in western Japan until March in 2011, the end of winter (Table 1 and Fig. 2). Western Japan is known as an overwintering area for migrating water birds flying from their nesting lakes in Siberia. Migrating and resident birds share living territories during winter in these areas, which explains the reason why resident birds, such as raptors and mandarin ducks with H5N1 viruses in Groups B and C were found in addition to infected migratory birds

Table 1. The list of H5N1 HPAIVs isolated in the definitive diagnosis at Tottori University

Sampling day	Bird species	City, Prefecture	Strain name of the isolate	HA gene	
				Accession number	Group
December, 2010					
4th	Tundra swan	Yonago, Tottori	Tundra swan/Tottori/12-002/2010 (H5N1) ^{b)}	AB677923	C
15th	Hooded crane	Izumi, Kagoshima	Hooded crane/Kagoshima/1/2010 (H5N1)	AB760239	C
18th	Hooded crane	Izumi, Kagoshima	Hooded crane/Kagoshima/2/2010 (H5N1)	AB760247	C
20th	Hooded crane	Izumi, Kagoshima	Hooded crane/Kagoshima/4612J001/2010 (H5N1)	AB760255	C
	Hooded crane	Izumi, Kagoshima	Hooded crane/Kagoshima/4612J003/2010 (H5N1)	AB760263	C
21th	Hooded crane	Izumi, Kagoshima	Hooded crane/Kagoshima/4612J004/2010 (H5N1)	AB760271	C
24th	Hooded crane	Izumi, Kagoshima	Hooded crane/Kagoshima/4612J008/2010 (H5N1) ^{b)}	AB677931	C
January, 2011					
12th	Common pochard	Itami, Hyogo	Common pochard/Hyogo/2801B004/2011 (H5N1)	AB747123	B
14th	Tufted duck	Matsue, Shimane	Tufted duck/Shimane/5501A008/2011 (H5N1)	AB747164	B
19th	Black headed gull	Yonago, Tottori	Black headed gull/Tottori/1-009/2011 (H5N1)	AB747167	B
24th	Tufted duck	Yonago, Tottori	Tufted duck/Tottori/1-012/2011 (H5N1)	AB747169	B
25th	Little grebe	Itami, Hyogo	Little grebe/Hyogo/2801B015/2011 (H5N1)	AB747127	B
26th	Mandarin duck	Niyodogawa, Kochi	Mandarin duck/Kochi/3901C005/2011 (H5N1) ^{b)}	AB677891	C
31st	Mandarin duck	Nagasaki, Nagasaki	Mandarin duck/Nagasaki/4201A011/2011 (H5N1)	AB747105	C
	Mandarin duck	Nagasaki, Nagasaki	Mandarin duck/Nagasaki/4202A012/2011 (H5N1)	AB747107	C
February, 2011					
1st	Mandarin duck	Nobeoka, Miyazaki	Mandarin duck/Miyazaki/22M686/2011 (H5N1)	AB747131	C
	Tufted duck	Daisen, Tottori	Tufted duck/Tottori/3102S004/2011 (H5N1)	AB747111	B
	Tufted duck	Matsue, Shimane	Tufted duck/Shimane/5502B018/2011 (H5N1)	AB747171	B
2nd	Peregrine falcon ^{a)}	Saito, Miyazaki	Peregrine falcon/Miyazaki/22M684/2011 (H5N1)	AB747135	C
3rd	Common pochard	Yonago, Tottori	Common pochard/Tottori/3102S012/2011 (H5N1)	AB747114	B
4th	Mandarin duck	Nagasaki, Nagasaki	Mandarin duck/Nagasaki/4202A023/2011 (H5N1) ^{b)}	AB677867	C
6th	Tufted duck	Ube, Yamaguchi	Tufted duck/Yamaguchi/3502B007/2011 (H5N1) ^{b)}	AB677851	C
	Peregrine falcon ^{a)}	Yonago, Tottori	Peregrine falcon/Tottori/3102S019/2011 (H5N1)	AB747117	B
7th	Mandarin duck	Nakatsu, Oita	Mandarin duck/Oita/4402F038/2011 (H5N1)	AB747138	C
8th	Mandarin duck	Nakatsu, Oita	Mandarin duck/Oita/4402F041/2011 (H5N1)	AB747141	C
	Mandarin duck	Nakatsu, Oita	Mandarin duck/Oita/4402F042/2011 (H5N1)	AB747144	C
	Ural owl	Naga, Tokushima	Owl/Tokushima/3602A023/2011 (H5N1) ^{b)}	AB677883	B
	Little grebe	Morotsuka, Miyazaki	Little grebe/Miyazaki/22M718/2011 (H5N1)	AB747147	C
	Common pochard	Matsue, Shimane	Common pochard/Shimane/5502B024/2011 (H5N1) ^{b)}	AB677843	B
9th	Mandarin duck	Oita, Oita	Mandarin duck/Oita/4402B056/2011 (H5N1)	AB677859	C
	Tufted duck	Izumo, Shimane	Tufted duck/Shimane/3202C002/2011 (H5N1)	AB747173	B
11th	Mute swan	Kato, Hyogo	Mute swan/Hyogo/2802F001/2011 (H5N1)	AB747109	B
	Peregrine falcon	Miyazaki, Miyazaki	Peregrine falcon/Miyazaki/22M754/2011 (H5N1)	AB747150	C
12th	Peregrine falcon	Isahaya, Nagasaki	Peregrine falcon/Nagasaki/4202A039/2011 (H5N1)	AB747153	C
13th	Hooded crane	Izumi, Kagoshima	Hooded crane/Kagoshima/110214-3/2011 (H5N1)	AB760279	C
14th	Mandarin duck	Nichinan, Miyazaki	Mandarin duck/Miyazaki/22M765/2011 (H5N1)	AB747155	C
15th	Grey heron	Oita, Oita	Grey heron/Oita/4402B069/2011 (H5N1)	AB747159	C
	Peregrine falcon ^{a)}	Nobeoka, Miyazaki	Peregrine falcon/Miyazaki/22M771/2011 (H5N1)	AB747161	C
16th	Peregrine falcon	Seika, Kyoto	Peregrine falcon/Kyoto/2602A009/2011 (H5N1) ^{b)}	AB677907	B
17th	Peregrine falcon ^{a)}	Kasugai, Aichi	Peregrine falcon/Aichi/2302O017/2011 (H5N1) ^{b)}	AB677915	C
18th	Mandarin duck	Miyazaki, Miyazaki	Mandarin duck/Miyazaki/22M807-1/2011 (H5N1) ^{b)}	AB677875	C
22nd	Great crested grebe	Nishinomiya, Hyogo	Great crested grebe/Hyogo/2802E082/2011 (H5N1) ^{b)}	AB677899	B
March, 2011					
6th	Tufted duck	Matsue, Shimane	Tufted duck/Shimane/3203A004/2011 (H5N1)	AB747119	B

a) These birds were applied for autopsy in this study. b) Sakoda *et al.* [15].

(Fig. 1). Moreover, HPAI outbreaks in poultry were dominantly observed in the western area. Resident birds might play a role as intermediate hosts between chicken populations and water birds. In early spring, the late phase of out-

breaks, the viruses in Group C were isolated in the Honsyu region (Fig. 2). The spreading of the Group C viruses might be due to migrating or wandering birds that are returning to northern breeding sites.

Fig. 1. Phylogenetic trees of the HA genes of H5N1 HPAIV isolated in 2010–11 winter in Japan and Korea. The isolates were divided into three (A–C) groups. (a) Groups A–B; (b) Group C. The full protein coding regions of H5 HA genes were used for the analysis. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Numbers at the nodes indicate confidence levels in a bootstrap analysis with 1,000 replications. Bootstrap values of more than 60 are shown at each branch. The host and year in strain names were abbreviated as follows: chicken; Ck, duck; Dk, 2011; 11. The viruses isolated from domestic poultry are underlined. The strains isolated from birds of prey are denoted by an asterisk. Blue indicates the viruses isolated in Korea. Of these, isolates in south-middle region were additionally in italic with boldface [8, 9].

Fig. 2. Map of H5N1 virus infection in wild birds (circles) and chickens (triangles) in the 2010–11 winter in Japan. The times of HPAI outbreaks were divided into 6 separate terms, represented by the different colors. Subsequent cases at the same places and in the same period are omitted.

H5N1 HPAIVs isolated in Korea around the same period [9] were classified into Group C (Fig. 1b). Kim *et al.* reported that virus isolates from poultry in Korea fell into two sublineages, south-middle and north-middle, which were distinct geographic regions in the HPAI outbreaks in poultry, but the isolates from wild birds were not subgrouped in the phylogenetic tree, which displays only topology [9]. Interestingly, some Korean viruses isolated in the south-middle region and some viruses in Yamaguchi prefecture and the Kyusyu region of Japan were classified into the same subgroups in the present study (C-2, C-3 and C-4 in

Fig. 1b). Because these regions are geographically close to each other, migratory birds were suspected of contributing to the transportation of the viruses across the sea. On the other hand, all the viruses in Subgroups C-1 and C-6 were isolated only in the Kyusyu region (Fig. 1b), indicating that these viruses were maintained in local bird populations during the outbreak periods. Moreover, the viruses in these subgroups were isolated from poultry in addition to resident birds, suggesting that direct or indirect viral transmission among them occurred in each prefecture.

All the isolates from hooded cranes at the Izumi migra-

Table 2. Viral titers recovered from the tissues of the raptors died of H5N1 HPAIV infection

Samples	Peregrine falcons				Northern goshawk
	Miyazaki 22M684	Shimane 3102S019	Miyazaki 22M771	Aichi 2302O017	Tochigi 64
Organs					
Brain	5.7	7.5	5.7	5.5	4.7
Trachea	- a)	5.3	-	3.7	2.5
Lung	3.7	3.7	4.3	4.3	4.3
Liver	-	4.5	-	2.5	-
Pancreas	-	-	3.3	≤2.0	3.3
Spleen	-	-	-	≤2.0	-
Heart	-	2.5	-	2.7	-
Kidney	-	3.3	2.7	5.3	-
Locomotorium					
Breast muscle	-	2.5	-	-	-
Digestive organs					
Esophagus	-	4.7	2.5	3.7	≤2.0
Avian crop	-	4.5	-	3.7	-
Proventriculus	-	2.3	-	3.5	≤2.0
Gizzard	-	2.7	-	2.5	≤2.0
Duodenum	-	-	-	2.5	≤2.0
Jejunum-ileum	-	-	-	4.5	-
Colon	2.5	4.5	4.5	4.5	≤2.0
Cloaca	2.7	4.3	3.3	4.5	3.3

a) ≤1.5.

tion grounds in Kagoshima prefecture were classified into Subgroup C-5. The viruses were isolated in December 2010 and February 2011 (Table 1). These results suggest that the viruses were present in the crane populations for 2 months. Hooded crane is evaluated as Vulnerable on the IUCN (International Union for Conservation of Nature) Red List of Threatened Species. Of a total population of about 10,000 birds, 90% live at high densities and overwinter at the Izumi crane migration grounds. In the HPAI outbreaks in 2010–11, H5N1 HPAIV infection was confirmed in only seven cranes (Table 1), suggesting that the pathogenicity of circulating HPAIV to these birds was low. To protect cranes from H5N1 HPAIV infection in the future, the number of overwintering cranes and the antibody prevalence in these birds should be continuously monitored.

In the outbreaks in Japan, quite a few raptors died of HPAIV infection [15]. H5N1 HPAIVs were also reported to be lethal to other raptor species such as American Kestrel (*Falco sparverius*) [4], Crested Hawk-eagle (*Nisaetus cirrhatus*) [22], Eurasian Sparrowhawk (*Accipiter nisus*) [3], Gyr Falcon-Saker hybrids (*Falco rusticolus* × *Falco cherrug*) [1] and Saker Falcon (*Falco cherrug*) [10]. Gyr Falcon-Saker hybrids can also be infected by feeding on infected prey [1]. Furthermore, viruses were recovered from digestive organs of some raptors dissected in this study (Table 2). Together, these results suggest that raptors were infected with HPAIV by their predatory behavior, eating infected birds or carrion in the environment. Since viruses were disseminated to systemic tissues of the dissected raptors (Table 2), they were a potential source of infection to other scavengers.

The past HPAI outbreaks in Japan were successfully con-

trolled by stamping out measures without the use of vaccine. As of December 2012, no outbreak had been reported since March 2011. HPAIV should be contained in domestic poultry population and eradicated by proper countermeasures as described previously [15], and this should help to protect wild fowls including rare species from HPAIV infection. Moreover, it is important that all diagnosis institutes rapidly report information about outbreaks and causal viruses to the public in order to take preemptive measures against further HPAI occurrences.

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